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EXH. B

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Liposidomycin C Inhibits Phospho-*N*-acetylmuramyl-pentapeptide Transferase in Peptidoglycan Synthesis of *Escherichia coli* Y-10

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Liposidomycin C ($C_{42}H_{67}N_5O_{21}S$, M.W. 1009) is a novel nucleoside antibiotic containing uracil, a sulfated aminosugar, and a fatty acid. It is a specific inhibitor of peptidoglycan synthesis of bacteria, inhibiting the formation of the lipid intermediates from uridine 5'-diphospho-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-[^{14}C]alanyl-D-[^{14}C]alanine and uridine 5'-diphospho-*N*-acetylglucosamine with a particulate enzyme from *Escherichia coli* Y-10. It also inhibited the formation of MurNAc(-pentapeptide)-P-P-lipid in the absence of UDP-GlcNAc. On the other hand, it inhibited the activity of *N*-acetylglucosamine transglycosylase and peptidoglycan transglycosylase only weakly using the same system from *E. coli*. Thus, it is concluded that the site of action of liposidomycin C is phospho-*N*-acetylmuramyl-pentapeptide transferase in peptidoglycan synthesis.

In screening for inhibitors of bacterial peptidoglycan synthesis, liposidomycins were found in the culture filtrate and mycelia of *Streptomyces griseosporeus*.[1] These liposidomycins were found to contain at least twelve active components. Three major components, liposidomycins A, B, and C have been isolated and their physico-chemical properties reported.[1] Recently the structures of liposidomycins B and C were elucidated.[2,3] They are novel lipid-containing uracil nucleosides of unusual complexity as shown in Fig. 1. Tunicamycin, a fatty acyl nucleoside antibiotic, also inhibited peptidoglycan synthesis and the site of action was reported to be the inhibition of the formation of lipid inter-

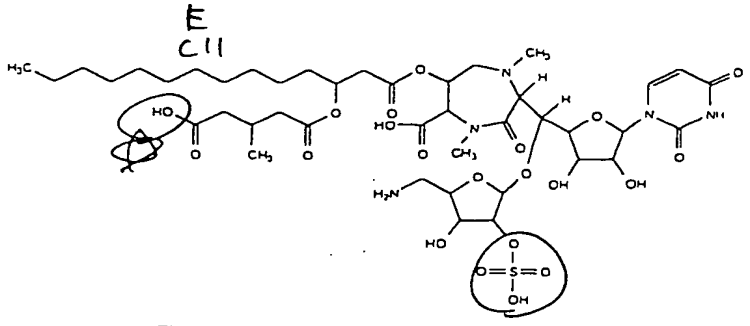

Fig. 1. Structure of Liposidomycin C.

Abbreviations: UDP-MurNAc-pentapeptide, Uridine 5'-diphospho-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine; UDP-GlcNAc, Uridine 5'-diphospho-*N*-acetylglucosamine.

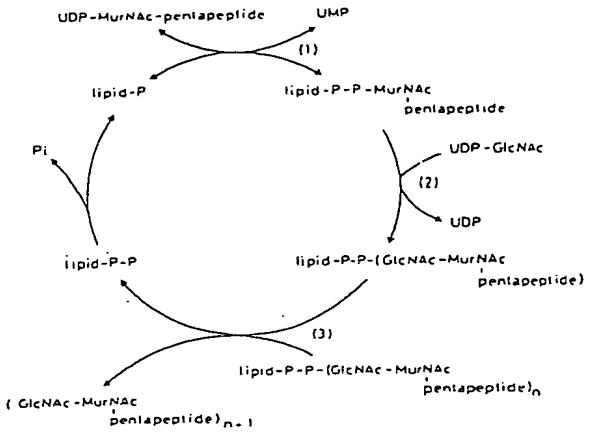

Fig. 2. Pathway of Peptidoglycan Synthesis from UDP-MurNAc-pentapeptide and UDP-GlcNAc with a Particulate Enzyme of *E. coli*.

(1) phospho-*N*-acetylmuramyl-pentapeptide transferase. (2) *N*-acetylglucosamine transglycosylase. (3) peptidoglycan transglycosylase. The pathway was the revised one from ref. 6.

mediates.[4,5] It was found that liposidomycin C inhibited *in vitro* peptidoglycan synthesis of *E. coli* Y-10 having an activity with a magnitude three orders higher than that of tunicamycin.

This paper describes the site of action of liposidomycin C in peptidoglycan synthesis of *E. coli* Y-10. The primary site of action of liposidomycin C is found to be phospho-MurNAc-pentapeptide transferase, the first step of the lipid cycle of peptidoglycan synthesis in bacteria, as shown in Fig. 2.[6]

Materials and Methods

Antibiotics. Liposidomycin C was isolated as previously reported.[1] Tunicamycin, vancomycin, and ristocetin were purchased from the Sigma Chemical Company, St. Louis, U.S.A. Enramycin was a gift from Takeda Chemical Industries, Ltd. Osaka, Japan.

Radiochemicals. UDP-[U-^{14}C]GlcNAc (302 mCi/mmol) was purchased from Amersham. UDP-MurNAc-L-Ala-D-Glu-*meso*-Dap-D-[^{14}C]Ala-D-[^{14}C]Ala was prepared as previously described.[7]

Organisms and growth conditions. Bacillus cereus T and *Escherichia coli* Y-10 were grown in bouillon medium (Eiken Chemical Co., Ltd.) at 37°C on a rotary shaker.

Preparation of particulate enzyme of E. coli Y-10. Particulate enzyme was prepared by grinding cells of *E. coli* Y-10 with sea sand (20~35 mesh) as previously reported.[7]

Preparation of UDP-MurNAc-L-Ala-D-Glu-meso-Dap-D-Ala-D-Ala. UDP-MurNAc-pentapeptide was obtained after inducing its accumulation in cells of *B. cereus* T by treatment with 12.5 μg of vancomycin per ml as previously reported.[7] From 10 l of the culture, approximately 600 OD units (A_{260}) of UDP-MurNAc-pentapeptide were isolated.

Assay of step 1 [phospho-MurNAc-pentapeptide transferase (EC 2.7.8.13)] in peptidoglycan synthesis from UDP-MurNAc-pentapeptide and UDP-GlcNAc with a particulate enzyme from E. coli Y-10. The assay was done using a particulate enzyme prepared from *E. coli* Y-10 by a simple modification of the previous method.[8] A reaction mixture (25 μl) containing 100 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, *D*-[^{14}C]Ala-labeled UDP-MurNAc-pentapeptide (20,000 cpm), 0.1 mM UDP-GlcNAc (Sigma), 25 μl of antibiotic or distilled water, and 10 μl of particulate enzyme (protein concentration 15 mg/ml) was incubated for 10~60 min at 37 C. The reaction was stopped by the addition of 25 μl of 6 M pyridinium acetate (pH 4.2), and the lipid intermediates in the mixture were extracted twice with 100 μl of *n*-butanol.[9] The extract was then transferred to a scintillation vial. The radioactivity was measured using a scintillation fluid, Pico-Fluor (Packard) with a liquid scintillation counter (lipid intermediates accumulation, step(s) 1 or 1 and 2).

The peptidoglycan that remained in the water layer was precipitated by adding an excess of 5% trichloroacetic acid. The precipitate was collected on a Whatman membrane filter GF/C, and washed twice with an excess of 5% trichloroacetic acid. The radioactivity of the precipitate on a filter was counted using a scintillation fluid, Filter-Count (Packard) with a liquid scintillation counter (peptidoglycan synthesis, steps 1, 2, and 3).

Assay of step 2 [GlcNAc transglycosylase] in peptidoglycan synthesis from UDP-MurNAc-pentapeptide and UDP-GlcNAc with a particulate enzyme from E. coli Y-10. A reaction mixture without UDP-GlcNAc (2.5 μl of 1 M Tris-HCl (pH 7.5), 2.5 μl of 0.2 M $MgCl_2$, 5 μl of 2 mM UDP-MurNAc-pentapeptide, 5 μl of particulate enzyme, and 2.5 μl of distilled water) was incubated for 30 min at 37°C to accumulate MurNAc(-pentapeptide)-P-P-lipid, and then 5 μl of UDP-[U-^{14}C]GlcNAc (20,000 cpm) and 2.5 μl of antibiotic or distilled water was added. After additional incubation of the reaction mixture for 10 min, lipid intermediates were assayed as described above.

Assay of step 3 [peptidoglycan transglycosylase (EC 2.4.1.129)] in peptidoglycan synthesis from UDP-MurNAc-pentapeptide and UDP-GlcNAc with a particulate enzyme

from E. coli Y-10. A reaction mixture (2.5 μl of 1 M Tris-HCl (pH 7.5), 2.5 μl of 0.2 M $MgCl_2$, 5 μl of 2 mM UDP-MurNAc-pentapeptide, 5 μl of UDP-[U-^{14}C]GlcNAc (20,000 cpm), 5 μl of particular enzyme, and 2.5 μl of distilled water) was incubated for 5~20 min at 37°C to accumulate GlcNAc-MurNAc(-pentapeptide)-P-P-lipid. After incubation, 2.5 μl of antibiotic or distilled water was added and the reaction mixture was incubated for 120 min at 37°C. The reaction mixture was extracted with *n*-butanol as described above and the water layer was spotted on Whatman 3MM paper. After ascending paper chromatography with isobutyric acid–1 N NH_4OH (5:3), spots on the paper corresponding to peptidoglycan (R_f=0) were cut out. The radioactivity was measured as described above.

Results and Discussion

The effects of liposidomycin C on the formation of lipid intermediates and peptidoglycan have been examined with this system. The courses of lipid intermediate accumulation and peptidoglycan synthesis from D-[^{14}C]Ala-labeled UDP-MurNAc pentapeptide and UDP-GlcNAc in the presence or the absence of liposidomycin C are shown in Fig. 3. With and without UDP-GlcNAc, formation of lipid intermediates and peptidoglycan were inhibited by liposidomycin C. This suggests

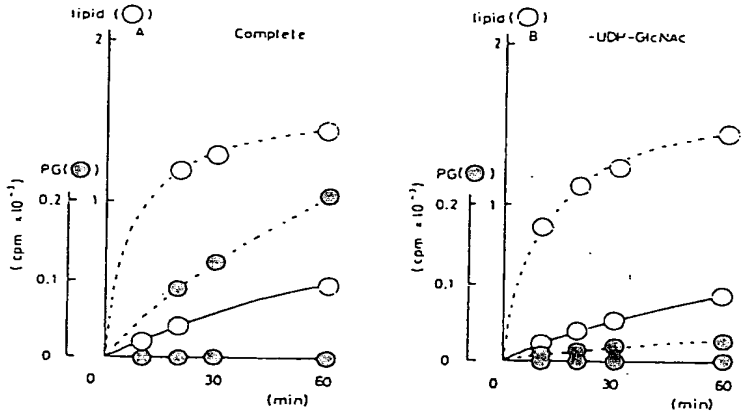

Fig. 3. Effects of Liposidomycin C on Lipid Intermediates and Peptidoglycan Syntheses by a Particulate Enzyme of *E. coli* Y-10.

Lipid intermediates and peptidoglycan were measured by the method described in Materials and Methods. Complete reaction mixture (A) and reaction mixture without UDP-GlcNAc (B). ○, lipid intermediate; ◎, peptidoglycan; ------, no antibiotic; ——, liposidomycin C (1 μg/ml).

Table I. Effects of Liposidomycin C on Phospho-*N*-acetylmuramyl-pentapeptide Transferase Activity of a Particulate Enzyme of *E. coli* Y-10

A reaction mixture (50 μl) containing 100 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, D-[^{14}C]Ala-labeled UDP-MurNAc-pentapeptide (20,000 cpm), 0.1 mM UDP-GlcNAc, 5 μl of liposidomycin C at various concentrations, and 5 μl of particulate enzyme was incubated for 10 min at 37°C. Other methods are described in Materials and Methods.

Antibiotics	Concentration (μg/ml)	MurNAc(-pentapeptide)-P-P-lipid formation cpm (inhibition %)	
		−UDP-GlcNAc	+UDP-GlcNAc
None	0	367 (0)	477 (0)
Liposidomycin C	0.025	241 (34)	333 (30)
	0.05	36 (90)	41 (91)
	0.1	17 (95)	31 (94)
	0.2	25 (93)	22 (95)
	1	21 (94)	9 (98)

Table II. EFFECTS OF LIPOSIDOMYCIN C ON *N*-ACETYLGLUCOSAMINE TRANSGLYCOSYLASE ACTIVITY OF A PARTICULATE ENZYME OF *E. coli* Y-10

A reaction mixture (2.5 μl of 1 M Tris-HCl (pH 7.5), 2.5 μl of 0.2 M $MgCl_2$, 5 μl of 2 mM UDP-MurNAc-pentapeptide, 5 μl of particulate enzyme, and 2.5 μl of distilled water) was incubated for 30 min at 37 C (1st incubation). Then UDP-[U-^{14}C]GlcNAc (20,000 cpm) was added with and without antibiotic, and the reaction mixture was incubated for 10 min at 37 C (2nd incubation). Other methods are described in Materials and Methods.

Antibiotics	Concentration (μg/ml)	GluNAc-MurNAc(-pentapeptide)-P-P-lipid formation cpm (inhibition %)
None	0	1511 (0)
Liposidomycin C	0.1	1086 (28)
	1	921 (39)
Tunicamycin	10	946 (37)
	100	1101 (27)
Enramycin	500	419 (72)

that liposidomycin C inhibits step 1 thus inhibiting peptidoglycan synthesis.

Inhibitory effects of liposidomycin C at various concentration on phospho-MurNAc-pentapeptide transferase (step 1) were examined with and without UDP-GlcNAc. As shown in Table I, liposidomycin C inhibits 50% of phospho-MurNAc-pentapeptide transferase activity at 0.03 μg/ml in both reactions. It also inhibits peptidoglycan synthesis at 0.038 μg/ml which is comparable to phospho-MurNAc-pentapeptide transferase inhibition in the same system.

Next, we tested the effects of liposidomycin C on GlcNAc transglycosylase (step 2). After the formation of MurNAc(-pentapeptide)-P-P-lipid from UDP-MurNAc-pentapeptide in the absence of UDP-GlcNAc, liposidomycin C and the substrate (UDP-[^{14}C]GlcNAc) were added. Formation of ^{14}C-labeled GlcNAc-MurNAc(-pentapeptide)-P-P-lipid from MurNAc(-pentapeptide)-P-P-lipid and UDP-[^{14}C]-GlcNAc was completed after a 10-min incubation period. The butanol layer containing ^{14}C-labeled GlcNAc-MurNAc(-pentapeptide)-P-P-lipid was then counted. Liposidomycin C did not inhibit step 2 strongly at 1 μg/ml (39% inhibition), but enramycin (step 2 inhibitor)[10] showed 72% inhibition at 500 μg/ml (Table II). Tunicamycin (step 1 inhibitor) also did not show inhibition in step 2 strongly at 100 μg/ml (27% inhibition).

Table III. EFFECTS OF LIPOSIDOMYCIN C ON PEPTIDOGLYCAN TRANSGLYCOSYLASE ACTIVITY OF A PARTICULATE ENZYME OF *E. coli* Y-10

A reaction mixture (2.5 μl of 1 M Tris-HCl (pH 7.5), 2.5 μl of 0.2 M $MgCl_2$, 5 μl of 2 mM UDP-MurNAc-pentapeptide, 5 μl of UDP-[U-^{14}C]GlcNAc (20,000 cpm), 5 μl of particulate enzyme, and 2.5 μl of distilled water) was incubated for 5 min* at 37 C (1st incubation). Then 2.5 μl of antibiotic or distilled water was added, and the reaction mixture incubated for 2 hr at 37 C (2nd incubation). Other methods are described in Materials and Methods.

	Peptidoglycan synthesis, cpm (inhibition %)
1st incubation	45
2nd incubation	
No antibiotic	364 (0)
Liposidomycin C (1 μg/ml)	230 (42)
Vancomycin (100 μg/ml)	70 (92)
Ristocetin (100 μg/ml)	50 (98)

* Similar results were obtained with 10 or 20 min of incubation.

We believe that the inhibition of over-all reactions is caused by the step 1 inhibition of liposidomycin C.

In the presence of enramycin (100 μg/ml), lipid compounds (compound X) accumulated in the aqueous phase when the reaction mixture were extracted with *n*-butanol at pH 4.2.[10] But liposidomycin C (1 μg/ml) and tu-

nicamycin (100 μg/ml) inhibited both water-soluble and butanol-soluble lipid intermediates (data not shown).

Next, we tested the effects of liposidomycin C on peptidoglycan transglycosylase. After the formation of GlcNAc-MurNAc(-pentapeptide)-P-P-lipid from UDP-MurNAc-pentapeptide and UDP-[^{14}C]GlcNAc incubation (5~20 min), liposidomycin C was added and the formation of peptidoglycan was measured. Liposidomycin C slightly inhibited the peptidoglycan transglycosylase at 1 μg/ml (30~40% inhibition). In contrast, the peptidoglycan transglycosylase inhibitors vancomycin[11] and ristocetin[12] were found to inhibit this reaction completely at 100 μg/ml (90~100% inhibition, Table III). Vancomycin and ristocetin did not inhibit step 1 at 100 μg/ml (data not shown).

It was already known that peptidoglycan transglycosylase was identical with the penicillin binding protein 1B.[13,14] Vancomycin and ristocetin were also inhibitors of penicillin binding protein 1B transglycosylase.

From the data described above, it is concluded that the primary site of action of liposidomycin C is phospho-*N*-acetylmuramyl-pentapeptide transferase (step 1), the first step of lipid cycle in peptidoglycan synthesis of bacteria. It is known that tunicamycin[4,5] and amphomycin[15] also inhibit the same step, but liposidomycin C has been shown to be the most potent inhibitor (ID_{50} of tunicamycin was about 12 μg/ml in contrast to 0.038 μg/ml for liposidomycin C in our conditions).[4,15] In contrast to the high *in vitro* activity, the antibacterial activity of liposidomycin C is limited, as reported previously.[1] The reason has yet to be determined.

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